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Central adenosine A_1 and A_{2A} receptors mediate the antinociceptive effects of neuropeptide S in the mouse formalin test

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ABSTRACT

Aims: The present study aimed to investigate the intraplantar (ipl) and central (icv) effects of neuropeptide S (NPS) in the formalin test and to evaluate the role of adenosine receptors, mainly A_1 and A_{2A}, in mediating such effects.
Main methods: The ipl injection of formalin was used to assess the nociceptive activity. Moreover, by pretreating mice with non-selective and selective antagonists of adenosine receptors, the effects of icv NPS on formalin-induced ongoing nociception were assessed.
Key findings: Morphine-induced antinociceptive effects were observed during phases 1 and 2 of the test, while indomethacin was active only at the later nociceptive phase. The ipl injection of NPS (alone or combined with formalin) did not modify the nociceptive response. However, icv NPS significantly reduced formalin-induced nociception during both phases. Caffeine (3 mg/kg, ip), a non-selective adenosine receptor antagonist, prevented NPS-induced antinociceptive effects. Similar to caffeine, icv ZM241385 (0.01 nmol), an A_{2A} receptor antagonist, prevented the antinociceptive effects of NPS. Moreover, icv DPCPX (0.001 nmol), an A_1 receptor antagonist, blocked the effects of NPS only during phase 1.
Significance: The above findings suggest that: (i) NPS evokes central antinociceptive effects by activating both A_1 and A_{2A} receptors during phase 1, but (ii) only the adenosine A_{2A} receptor during phase 2 of the formalin test.

Introduction

Pain is a symptom of many clinical disorders that affect a large population of humans, and the relief of pain is mainly achieved with pharmacological agents. In humans, the treatment of pain commonly employs nonsteroidal anti-inflammatory drugs and opioids, despite the well-known adverse effects of these drugs, such as gastric ulcers, nephrotoxicity, and abuse liability [3,36]. Safer and more effective analgesic drugs are a major focus in current pharmaceutical research.

A new target for the development of innovative analgesic drugs is the peptidergic system of neuropeptide S (NPS). NPS is an eicosapeptide that is named due to the presence of a serine residue in its amino-terminal portion [39]. The NPS peptide binds to NPSR, a typical 7-transmembrane domain G protein-coupled receptor [39]. Studies using CHO and HEK293 cell lines expressing recombinant NPSR showed an intracellular increase in the concentrations of Ca^{+2}, cAMP and phosphorylated MAPK in response to NPS, suggesting that NPSR can be coupled with Gq and Gs [28,39].

The NPS precursor mRNA is expressed in areas restricted to the central nervous system, such as the sensory trigeminal nucleus and lateral parabrachial nucleus (neighbor to the locus coeruleus; [38]). The latter area has been involved in the regulation of autonomic functions and nociceptive processing. In fact, visceral afferent input from the nucleus of the solitary tract [14] and pain stimuli from the spinal cord [12] are conducted to the forebrain through neurons from the lateral parabrachial nucleus. The lateral parabrachial nucleus has been reported to send neuronal projections to many areas where NPSR mRNA is highly expressed, including the ventromedial and paraventricular hypothalamic nuclei, amygdala, and periaqueductal gray [12]. In addition, NPSR mRNA is highly expressed in the cerebral cortex, thalamus, hypothalamus and amygdala [38,39]. This pattern of NPSR distribution in the brain suggests the involvement of the peptidergic system in the

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regulation of various biological functions, including locomotion, anxiety, arousal and wakefulness, food intake, drug reward, memory processing, and nociceptive transmission. For a review of all the biological actions modulated by this peptidergic system see Guerrini et al. [13]. Despite the fact that intracerebroventricular (icv) administration of NPS evokes antinociception [21,26], little is known about the mechanisms by which the activation of NPSR receptor signaling induces pain relief. In this work we investigated the effects of NPS on formalin-induced nociceptive behaviors. In addition, we studied the involvement of adenosine A_1 and A_{2A} receptors in the antinociceptive actions of NPS.

Material and methods

Animals

Male Swiss mice weighing 28–35 g from our own breeding stock were housed in groups of 10–12 per cage (33 cm × 40 cm × 17 cm) with food and water *ad libitum*. Animals were kept under controlled temperature (23 ± 2 ºC) and a 12-h light/dark cycle (lights on at 06:00). All experiments were conducted in accordance with Brazilian law no. 11.794/2008 for the experimental use of animals. The protocol was approved by the Ethic Committee for Animal Use of Federal University of Rio Grande do Norte (Protocol no. 012/2011). This study is reported following the ARRIVE guidelines [17].

Drugs

Human NPS was synthesized by Dr. Guerrini (Department of Chemistry and Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy) and was solubilized in saline (NaCl 0.9%). Indomethacin (a non-steroidal anti-inflammatory drug), caffeine (nonselective A_1 and A_{2A} receptor antagonist), DPCPX (1,3-dipropyl-8-cyclopentylxanthine; selective A_1 receptor antagonist) and ZM241385 (4-(−2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol; selective A_{2A} receptor antagonist) were all purchased from Sigma-Aldrich (San Louis, MO, USA). Caffeine was dissolved in saline solution. The stock solutions of DCPX, ZM241385 and indomethacin were prepared in 100% dimethylsulfoxide (DMSO), stored at −4 °C, and dissolved in saline solution just before the experiments. The final concentration of DMSO in DPCPX and ZM241385 solutions did not exceed 0.1%, while the indomethacin solution contained 8.3% DMSO. The concentration of DMSO did not cause any detectable effect *per se*. Morphine (Hipolabor Farmaceutica Ltda., Sabará, MG, Brazil), an opioid analgesic drug, was dissolved in saline solution.

Treatments

Twenty microliters of 1% formalin was injected subcutaneously into the plantar surface (intraplantar administration, ipl) of the right hind paw of mice. The effects of indomethacin (10 mg/kg, ip) and morphine (5 mg/kg, sc) were evaluated by injecting the drugs 30 min before formalin.

To evaluate the possible effects of NPS on nociceptive responses induced by formalin, the peptide was injected either intracerebroventricularly (icv, 0.1 nmol, 5 min pretreatment) or intraplantarly (10 nmol/20 μl, co-injected with formalin). Icv NPS was injected at a rate of 2 μl/min in a total volume of 2 μl. The icv dose of NPS used herein was previously shown to evoke antinociceptive effects [26]. To investigate the involvement of adenosine A_1 and A_{2A} receptors on the antinociceptive effects of NPS in the formalin test, caffeine (3 mg/kg) was injected intraperitoneally (ip), in a volume of 10 ml/kg, 15 min before formalin. In another subset of animals, DPCPX (0.001 nmol; 1 μl, icv) or ZM241385 (0.01 nmol; 1 μl, icv) was given 10 min before formalin.

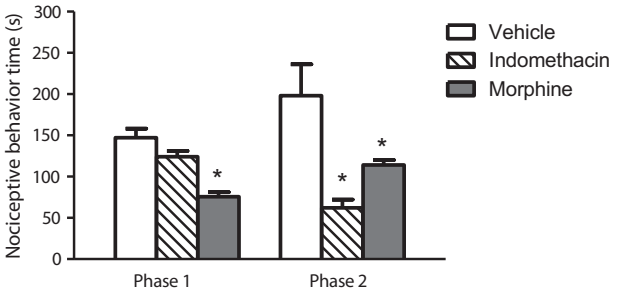

Fig. 1. Effects of standard analgesic drugs, indomethacin (10 mg/kg, ip) and morphine (5 mg/kg, ip) in the first (0–5 min) and second phases (15–30 min) of ongoing nociception induced by 1% formalin (20 μl/paw). Each bar represents the mean ± S.E.M. of 5–6 animals (ANOVA, Student–Newman–Keuls test, *P < 0.05 *vs.* control; phase 1: $F_{(2,14)} = 23.76$, phase 2: $F_{(2,14)} = 8.46$).

Cannula implantation in the lateral ventricle

All icv injections were performed by a 22-G guide cannula permanently implanted into the lateral ventricle. Surgical implantation of a stainless steel cannula into the lateral ventricle was conducted according to our previous studies [7]. Briefly, mice were anesthetized with a ketamine/xylazine solution (100 mg/kg and 10 mg/kg, ip, respectively) and placed in a stereotaxic apparatus. A vertical incision was made in the skin to expose the skull. A stainless steel guide cannula was implanted into the lateral ventricle and was fixed with dental cement. Coordinates toward the bregma were ML + 1.1 mm, AP −0.6 mm and DV −1.0 mm [25]. To prevent occlusion, a dummy cannula was inserted into the guide cannula. The dummy cannula protruded 1 mm from the guide cannula. After surgery, the animals were allowed to recover for at least 3 days.

Formalin test

The procedure was performed as previously described by Hunskaar and Hole [16]. The animals were acclimatized in the laboratory for at least 2 h before testing and then individually placed in a glass cone (20 cm in diameter) for 20 min. After the acclimatization period, formalin was ipl injected in the right hind paw of the mice. Immediately after the formalin injection, animals were put back into the glass cone and were observed for 30 min. A mirror was placed behind the glass cone to allow an unobstructed view of the formalin injected paw. The time (s) that animals spent licking, shaking and retracting the injected paw was timed with a chronometer and was considered to be indicative of ongoing nociception. The intraplantar formalin injection produced a

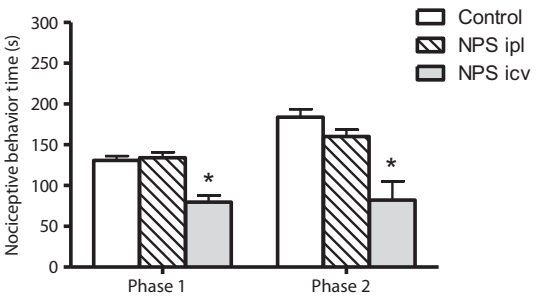

Fig. 2. Effects of NPS administration into the lateral ventricle (0.1 nmol) and the hind paw (10 nmol) in the first (0–5 min) and second phases (15–30 min) of ongoing nociception induced by 1% formalin (20 μl/paw). Each bar represents the mean ± S.E.M. of 6 animals. (ANOVA, Student–Newman–Keuls test, *P < 0.05 *vs.* control; phase 1: $F_{(2,16)} = 19.56$, phase 2: $F_{(2,16)} = 12.69$).

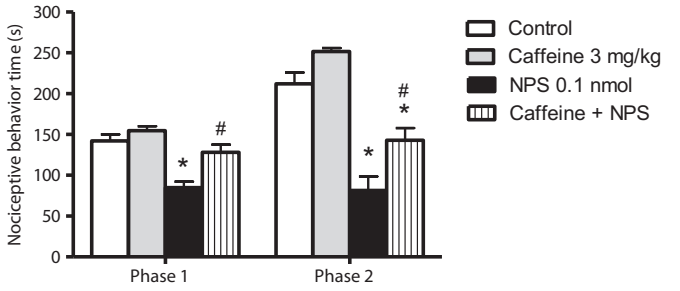

Fig. 3. Effect of pretreatment with caffeine (3 mg/kg, ip) on the antinociceptive actions of NPS (0.1 nmol, icv) in the first (0–5 min) and second phases (15–30 min) of ongoing nociception induced by 1% formalin (20 μl/paw). Each bar represents the mean ± S.E.M. of 6–8 animals. (ANOVA, Student–Newman–Keuls test, *P < 0.05 *vs.* control; #P < 0.05 *vs.* NPS; phase 1: $F_{(3,25)} = 14.26$, phase 2: $F_{(3,25)} = 23.10$).

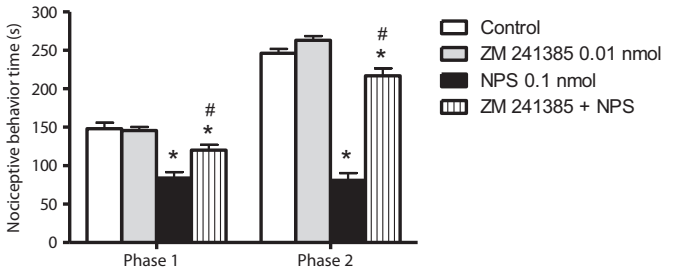

Fig. 5. Effect of pretreatment with ZM241385 (0.01 nmol, icv) on the antinociceptive actions of NPS (0.1 nmol, icv) in the first (0–5 min) and second phases (15–30 min) of ongoing nociception induced by 1% formalin (20 μl/paw). Each bar represents the mean ± S.E.M. of 6–8 animals. (ANOVA, Student–Newman–Keuls test, *P < 0.05 *vs.* control, #P < 0.05 vs. NPS; phase 1: P < 0.0001; $F_{(3,28)} = 14.19$; phase 2: P < 0.0001, $F_{(3,28)} = 70.73$).

biphasic nociceptive response: (I) an acute phase of short duration followed by (II) a longer lasting tonic phase. Hence, the evaluation of the nociceptive behavior was divided into two phases. The first 5 min after formalin injection was known as the first phase, followed by a quiescent period of approximately 10 min, and then the second phase occurred from 15 to 30 min after this period.

Histology

After completing the test, mice were euthanized with sodium thiopental (>100 mg/kg, ip) and injected icv with methylene blue dye (2 μl). Mice were perfused with saline solution and their brains were removed to verify the placement of the guide cannula. Only the data from those animals with dispersion of the dye throughout the ventricles were used for statistical analysis (these were approximately 95% of cannula implanted animals).

Statistical analysis

The data presented herein were reported as mean ± S.E.M. Results were submitted to Levene's test for homogeneity of variance and to Kolmogorov–Sminorv's test for normality. Treated and control groups were compared using one-way ANOVA followed by the Student–Newman–Keuls test. A value of $P < 0.05$ was considered to be significant. These analyses were performed by GraphPad InStat software, version 3.06 (La Jolla, CA, USA) and Statistica, version 5.1 (Tulsa, OK, USA).

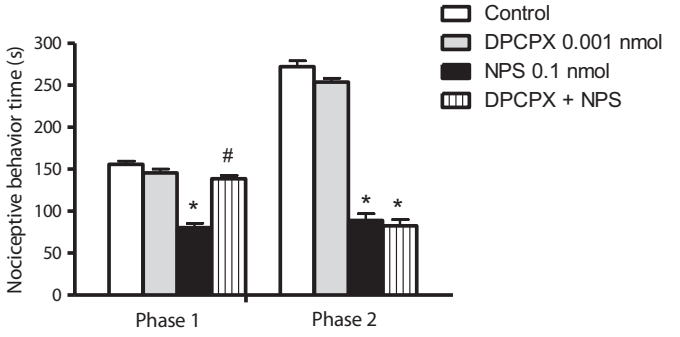

Fig. 4. Effects of pretreatment with DPCPX (0.001 nmol, icv) on the antinociceptive actions of NPS (0.1 nmol, icv) in the first (0–5 min) and second phases (15–30 min) of ongoing nociception induced by 1% formalin (20 μl/paw). Each bar represents the mean ± S.E.M. of 6–8 animals. (ANOVA, Student–Newman–Keuls test, *P < 0.05 *vs.* control, #P < 0.05 *vs.* NPS; phase 1: $F_{(3,26)} = 55.09$; phase 2: $F_{(3,26)} = 239.13$).

Results

Effects of standard antinociceptive drugs in the formalin test

Fig. 1 illustrates the effects of standard analgesic drugs, indomethacin and morphine on ongoing nociception induced by ipl formalin. Animals injected with formalin displayed a typical biphasic nociceptive response. Mice spent approximately 150 s displaying nociceptive behaviors during the first 5 min of the assay (phase 1), and approximately 200 s during the period of 15–30 min (phase 2). Pretreatment with morphine (5 mg/kg, sc) significantly attenuated the nociceptive behavior time in both phases of the formalin test. The inhibitory effect exerted by the alkaloid amounted to 50% and 40% of the control for phases 1 and 2, respectively (Fig. 1). Moreover, animals treated with indomethacin (10 mg/kg, ip) displayed a statistically significant reduction of nociceptive behavior only during phase 2. The inhibitory effect of indomethacin was 67% of control values (Fig. 1).

Effects of NPS in the formalin test

Ipl NPS (10 nmol/20 μl/paw) did not evoke any nociceptive behavior in mice observed during 5 min (data not shown). Additionally, when co-injected ipl with formalin, NPS did not affect the nociceptive behavior time during phases 1 and 2 of the test (Fig. 2). However, supraspinal administration of NPS decreased the ongoing nociception time by 39% in phase 1 and by 55% in phase 2 (Fig. 2) compared to control values.

Effects of adenosine antagonists on the antinociceptive actions of NPS in the formalin test

Fig. 3 illustrates the effects of systemic pretreatment with caffeine on mice icv injected with NPS in the formalin test. Pretreatment with caffeine did not modify phase 1 or 2 of formalin-induced nociception (Fig. 3). However, ip caffeine fully prevented the antinociceptive effects of NPS in phase 1 and attenuated the antinociceptive actions of the peptide in phase 2 (Fig. 3).

Fig. 4 shows the actions of icv DPCPX on the antinociceptive effects of NPS in the formalin test. The administration of DPCPX did not change the nociceptive behavior time induced by formalin in both phases (Fig. 4). Interestingly, DPCPX prevented the antinociceptive effects of NPS during phase 1, but not during phase 2 of the formalin test (Fig. 4).

The effects of the icv pretreatment with ZM241385 in mice supraspinally treated with NPS are depicted in Fig. 5. The administration of ZM241385 did not change the ongoing nociception induced by formalin (Fig. 5). However, icv ZM241385 significantly attenuated the antinociceptive effects of NPS during phases 1 and 2 of the formalin test (Fig. 5).

Discussion

This study reinforces the antinociceptive action of NPS in the mouse formalin test and, for the first time, indicates that systemic administration of caffeine (non-selective A_1/A_{2A} receptor antagonist) and central injections of DPCPX (selective A_1 receptor antagonist) and ZM241385 (selective A_{2A} receptor antagonist) inhibit antinociception induced by icv NPS, thus supporting a role for central A_1 and A_{2A} receptors in mediating NPS-induced pain relief.

The formalin test is among the most commonly used analgesiometric assays [11,37]. As nicely summarized by Barrot [1], the formalin injection produces a biphasic behavioral reaction with an initial phase within the first minutes post-injection followed by a quiescent period (approximately 10 min) and a second phase of nociceptive behaviors lasting 20–40 min. Phase 1 is related to the direct stimulation of nociceptors and is sensitive to local anesthetics and opiates, while phase 2 involves both inflammatory mechanisms and central sensitization within the dorsal horn [37]. Phase 2 responds to various drugs with established clinical analgesic action, such as opiates [11], steroidal or non-steroidal anti-inflammatory drug analgesics [16], N-methyl-D-aspartate antagonists [6], and gabapentin [34].

To validate our experimental conditions, the effects of morphine and indomethacin were assessed in mice ipl injected with formalin. In line with published findings, the present results showed that the systemic administration of morphine attenuated both nociceptive phases of the formalin test, while indomethacin reduced formalin-induced enduring nociception only during phase 2 of the test [15,16].

Similar to morphine, icv NPS reduced formalin-induced nociceptive behaviors in both phases of the test. The antinociceptive effects of icv NPS were observed at the same dose as previously described in mice by Peng et al. [26] in the formalin test, and by Li et al. [21] in the tail withdrawal and the hot-plate tests. Recently, Zhang et al. [43] showed that exogenous NPS (1 nmol) attenuated thermal hyperalgesia and mechanical allodynia in chronic constriction sciatic nerve injury in rats.

In contrast, the ipl administration of NPS was not able to modify the response to formalin. Interestingly, NPS immunoreactive cells are found in the skin, skin appendages and immune cells including splenic lymphocytes and pulmonary alveolar macrophages in pigs [40,41]. Regarding the NPSR, it is also expressed in the skin and immune cells, *i.e.* macrophages, neutrophils, and intraepithelial lymphocytes [27,35]. A growing body of *in vitro* and *in vivo* assays suggest a proinflammatory profile of action for NPS [27,41,42]. Although some findings support a role for NPS in inflammation, the present data suggests that the activation of NPSR in the periphery does not affect nociceptive transmission.

The present findings showed that the systemic treatment with caffeine prevented the antinociceptive effects of NPS during both phases of the formalin test. A similar pattern was found when mice were supraspinally treated with the selective A_{2A} receptor antagonist ZM241385. Our findings also demonstrated that A_1 receptor signaling is involved only on the actions of NPS during phase 1 of the formalin test.

The mechanism by which NPS evokes central antinociceptive effects is still unknown. However, NPSR antagonists, which are inactive in nociceptive animal models, prevent NPS-induced antinociceptive effects [21,26]. No behavioral differences between NPSR knockout and wild-type mice were found in the formalin test [33]. The inactivity of NPSR antagonists in animal models of pain and the absence of a nociceptive phenotype in NPSR knockout mice strongly suggest that NPSergic pathways do not tonically modulate pain transmission.

Regarding the involvement of other neurotransmitter systems on the antinociceptive effects of NPS, Peng et al. [26] showed that the systemic administration of naloxone, a non-selective opioid receptor antagonist, failed to inhibit the antinociceptive effects of NPS suggesting that NPS-induced antinociception does not engage opioidergic pathways. A growing body of evidence suggests the participation of adenosinergic signaling in NPS-induced biological actions. First, Lage et al. [18], using PCR experiments, showed alterations in mRNA transcript levels of NPS and NPSR in the rat hypothalamus and brainstem after acute and repeated caffeine treatments. Second, our research group has demonstrated inhibitory effects of caffeine and ZM241385 on NPS-induced hyperlocomotion [2]. Third, extracellular adenosine seems to mediate NPS-induced hyperlocomotion because the inhibition of ecto-5′-nucleotidase prevented the psychostimulant actions of NPS [24]. In addition, an Asn(107)Ile NPSR polymorphism has been described in humans. This polymorphism has functional consequences; NPS is approximately 10-fold more potent at the Ile107 than at the Asn107 NPSR ([28]). A recent study in humans investigated the impact of the NPSR Asn(107)Ile polymorphism on affect-modulated startle response [10]. The authors observed that Ile107 NPSR carriers showed an increased startle magnitude in response to neutral stimuli, while a decreased startle magnitude was observed in response to unpleasant stimuli in those subjects when they ingested caffeine. Altogether, these findings support a clear interplay between NPS and adenosinergic systems in modulating different biological functions.

No information is available about the nociceptive effects of selective adenosine receptor antagonists when supraspinally injected in rodents. In this study, we observed that at the doses tested, the selective antagonists DPCPX and ZM241385 did not affect mouse behavior in the formalin test. Regarding caffeine, in the present study no alterations in nociceptive behaviors were observed in mice systemically treated with this non selective adenosine antagonist. This observation is in line with previous literature data for rodents in the formalin test [9,22].

As far as the localization of NPSR and adenosine receptor modulating pain transmission is concerned, some information is present in the literature. Recently, an electrophysiological study showed that NPS controls amygdala output and pain-related affective behaviors through a direct activation of inhibitory intercalated neurons. Additionally, Peng et al. [26] showed an increase in c-Fos protein expression in the periaqueductal gray in formalin-injected mice after icv NPS. Some evidence of the expression of A_1 receptors and A_{2A} receptors in these brain areas are reported in the literature [8,23,29,31]. However, focused immunohistochemical studies are required for reinforcing these hypotheses. The involvement of other brain areas (*e.g.*, thalamic nuclei; [20,38]) cannot be ruled out in considering the interaction between adenosine and NPS in controlling pain transmission.

The results of analgesiometric assays can be biased by the effect of drugs on locomotor behavior. It is widely reported that NPS produces psychostimulant effects [4,5,19,30,32,39]. However, we have previously demonstrated that 0.1 nmol of NPS, the dose used in the present study, does not modify locomotor activity in mice [7]. This finding suggests that the inhibitory effect exerted by NPS in the formalin test is indeed a genuine antinociceptive action. The same can be said for the action of caffeine. In fact, the alkaloid at the dose employed in the present study does not affect locomotion in mice [2]. No information is available about the central effects of DPCPX and ZM241385 on spontaneous locomotion. However, no alteration in animal gross behavior, such as agitation or increased ambulation into the cylinder cone, was noticed during the formalin test in animals treated with these antagonists. Collectively, these observations suggest that neither NPS nor adenosine antagonists, alone or combined, affect mouse locomotion at the doses used herein.

Conclusion

Our data revealed that icv but not ipl NPS significantly inhibited both phases of formalin-induced nociceptive behaviors. Moreover, receptor antagonist studies demonstrate the involvement of adenosine in the antinociceptive effects of NPS. In particular, both A_1 and A_{2A} receptors seem to be involved in the action of NPS on phase 1 of the formalin test, while only A_{2A} receptors contribute to the NPS-induced antinociceptive actions during phase 2 of the assay.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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